

Using IBMR3 Monoclonal Antibodies for the Detection of the Expression Antigens Profile on 3T3 and HT29 Cancer Cell Line after Analysis

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Abstract: The detection of several types of cancer is possible by using monoclonal antibody (MAB). Studies have shown that different types of cancer have related specific abnormal proteins, which are identified by MAB. IBMR3 is a MAB which was raised against synthetic peptides that are associated with the selected amino acid sequence of human interleukin-4 receptors. **Aims:** The aim of this study was to evaluate the effectiveness of specific monoclonal IBMR3 antibodies expression in the cancer cell lines of 3T3 and HT29, in order to recognize specific antigen and make evaluation, using immunoblotting technique which is widely use in this filed. **Methods:** Protein extracts were extracted from these cancer cell lines and used for western blot. The Immunoblotting were consequently subjected to densitometry analysis using bio imaging machine. This bio imaging process will facilitate to measure the molecular weights, peak height and raw volume of the protein bands for the 3T3 and HT29 cancer cell lines, which helps in diagnosis of any pathogenic antigen. **Results:** The bands obtained from bio imaging were exposed on the PVDF membrane. In the 3T3 bio imaging process revealed four bands and molecular weight were 299.58, 87.90, 41.67 and 23.54 KDa. However, bio imaging results for HT29 revealed also four bands with molecular weights: 90.11, 41.31, 23.87 and 20.86 KDa. The results of Peak height densitometry for IBMR3 antigen bands for 3T3 were: 1856.985, 551.769, 394.164, and 216.185. However HT29 peak height results were: 281.544, 101.711.202.668, 757.213. The raw volume (amount of protein bands of IBMR3 Ag for 3T3) were: 1460168.75, 206078.47, 161406.89, and 219583.16. However HT29 raw volume results were: 110197.11, 76106.84, 98632.59, and 221395.34. Negative protein staining for 3T3 and HT29 cancer cell line bands were done by using mouse IgM serotype. IgM serotype was not indicated that means no specific antigens for negative control IgM. **Conclusion:** The bio imaging revealed different results of the expression profile in molecular weight, peak height and raw volume between the two cancer cell lines. The results from this study suggest that the IBMR3 antigens were differentially expressed in 3T3 and HT29 and the molecular weight is higher in the 3T3 than HT29 cancer cell lines. In future it will be beneficial to categorize and study the character analysis of the IBMR3 antigen and its prospective role in cancer cells.

Keywords: SDS-Page, MAB IBMR3, Immunoblotting, Bio imaging, HT29 (Human Colorectal) Carcinoma Cancer Cell Line, and 3T3 (Mouse Fibroblast) Cell Line.

1. INTRODUCTION

1.1. IBMR3 MAB

IBMR3 MAB was raised against synthetic peptides that similar to certain amino acid sequences in human IL-4R (Mat, 1992). Cytokines and their receptors are vital factors for tumour advancement. IL-4R is expressed in many human epithelial tumours. IL-6, IL-4 and IL-8 levels are increased among patients with prostate, breast or colon cancer. The significance of the IL-4R overexpression in epithelial cancer cells has not been elucidated.

Cancer is a disease of a cell or a group of cells that divides and replicates uncontrollably because of the accumulation of both epigenetic and/or genetic changes (Kumar *et al.*, 2013).

Monoclonal antibodies (MABs) are the protein molecules which were synthetically created from hybridoma cells (stable cell lines) by fusing antibody-producing cells from the immunized animals with cells that confer immortality and yield high antibody production (Köhler & Milstein, 1975) or by recombinant deoxyribonucleic acid (DNA) technology. MAB technology is an important development in

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creating specific serologic reagents within a large diversity of antigens to produce several types of highly specific and reproducible immunological assays for fast and accurate diagnosis (Deb *et al.*, 2013). Previous study illustrated that the hybridoma cell culture which yields the monoclonal antibodies shows the possibility of an unrestrained supply of reagent (Listek, *et al.*, 2000).

Cancer types depend on the origin site of the malignant cells, the histology, or cell analysis (grading), and the extent of the disease (staging). Cancers are classified into 3 main groups, namely, carcinomas, sarcomas, and leukemia's or lymphomas. Cancers that developed in epithelial tissues are called carcinomas, which are detected in approximately 90% of human cancers. Cancers that developed in connective tissues

are called sarcomas, which are rare in humans. Leukemias and lymphomas are cancers that developed in cells that normally mature in the bloodstream and in the lymphatic system respectively (Cooper, & Hausman, 2000).

1.2. Hybridoma cells

Hybridomas which resulted from the hybridization have the survival chance. Generally, the spleen lymphocyte has a limited life span. Therefore, any B-cells which do not merge with myeloma will die in the culture. The isolated hybridoma cells are examined for their specificity to the antigen. This is because each hybridoma originated from B-cell creates copies for only one antibody as in Figure 1. (Köhler & Milstein, 1975): (Michnick and Sidhu, 2008).

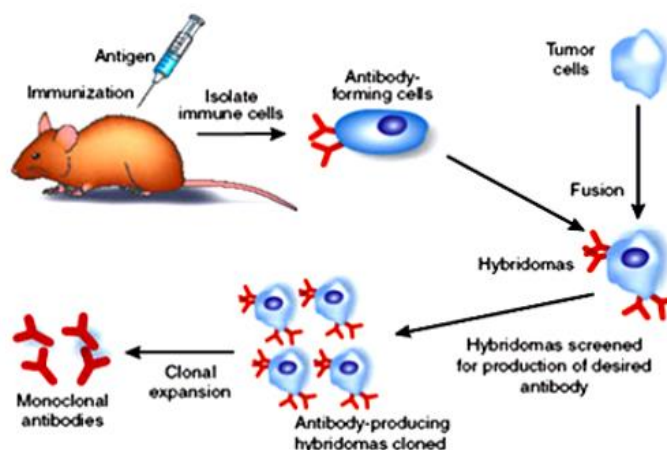


Figure 1: The production of monoclonal antibodies from a mouse spleen cell. Adapted from (Michnick and Sidhu, 2008)

Monoclonal antibodies are being increasingly used for therapy, laboratory research and diagnosis. It was suggested to use of synthetic peptides equivalent to amino acid sequence proteins to the raising numerous antibodies (Walter and Doolittle, 1983). Monoclonal antibodies (mAb or moAb) are monospecific antibodies that are the same because they are made by identical immune cells that are all clones of a unique parent cell, in contrast to *polyclonal antibodies* which are made from several different immune cells. Monoclonal antibodies have monovalent affinity, in that they bind to the same epitope. IBMR3 is a mAb of IgM Isotype produced in Advanced Medical and Dentil Institute / USM for research and a myeloma cell using special media (PEG) (poly ethylene glycol) and HAT (hypoxanthine Aminopterin Thymidine) (Hawkins *et al.*, 1992). (Hara and Mat, 2004) IBMR3 mAb provided evidences that might be familiar with the same epitope which is shared by molecules having different molecular masses.

MAB has ability to generate against all antigens target, purified and split into fragments. MAB has a special character to conjugate with radio nuclides, toxins, drugs or enzymes. By nature, mAb originate from one specific clone with higher specificity, purity, reliability and identify only one epitope of the antigen. However, these antibodies have need of secondary antibodies which are used in two analyses (Spinks, 2000). Such methods are used in laboratories for common techniques in many medical research and diagnostics (Hawkins *et al.*, 1992).

MAB has been widely used in immunotherapeutic because they assist with immune system cell molecules to produce anti-tumor responses and has the ability to increase the strength of immune reactions against tumor by Ligand formation with receptors on lymphocytes cells or antigen presenting cells (Murillo *et al.*, 2003). Antigen proteins which involved in the immune reaction can be separate via electrophoresis according to the molecular weight of their polypeptide chains (Shapiro *et al.*, 1967). To

confirm further the antibody specificity for the proteins western blotting is the most ideal technique practiced in Immunology and Microbiology (Stryer, 1996).

Only, previously produced by using synthetic peptides corresponding to selected amino acid sequences of the IL-4 receptor molecules (Mat, 1992); (Galizzi *et al.*, 1990). mAb can only be produced in certain strains of mouse or rat with histo-compatibility plasmacytoma fusion lines (Hara and Mat, 2004). These mice or rat have normal basal levels of IgM and of IgG isotopes with normal B and T-cell development (Molina *et al.*, 1996).

MAB, produced by B-cell clones of a single hybridoma or single parent cell line as a single type of antibody. In laboratory cultures media can produce hybridoma cell line from the fusion between one cell of normal B lymphocyte MAB are potentially competent of multiple functions. Successful as anticancer mAb has a special role in binding to an appropriate antigen in quantities adequate to mediate a disease-relevant response. Antibody has directly action power to destroy any target after conjugated radioactive isotopes or toxins, or antibody triggered apoptosis or indirect action power by activation of immune system components or blockade of critical receptors (Reichert and Valge-Archer, 2007).

1.3. Objective

The main aim of this study was to analyses the antigen specific expression of specific monoclonal IBMR3 antibodies in the cancer cell lines 3T3 and HT29 as main cells.

2. MATERIALS AND METHODS

2.1. Preparation of 3T3 (Mouse Fibroblast) Cell Line

The cells were harvested using cell dissociation solution 3ml / 25 cm² flask or 5 ml / 75 cm² flask for about 15-20 minutes for cells to dissociate and monitored under the microscope for floating cells.

The solution was decanted and the flask was tapped to lose the cells. Then 10 ml of phosphate buffer saline (PBS) sterile solution were added and flushed inside the flask bottom to lose the cells. The cells were pipetted into a 15 ml sterile falcon tube and spin under 2,000 rpm for 5 minutes and then removed the PBS. The cells were washed twice with PBS and centrifuged. Then, PBS removed and the cells were frozen at - 20° C.

2.2. Preparation of HT29 Cell Culture Human Colorectal Carcinoma Line

The cells were harvested using cell dissociation solution 3ml / 25 cm² flask or 5 ml / 75 cm² flask for about 15-20 minutes for cells to dissociate and

(monitor under the microscope for floating cells). The solution was decanted and the flask was tapped to lose the cells.

Then 10 ml PBS sterile solution were added and flushed inside the flask bottom to loosen the cells. The cells were pipetted into a 15 ml sterile falcon tube to spin under 2,000 rpm for 5 minutes and then PBS decanted. The cells were washed two times with PBS and centrifuged and then decanted again and the cell was kept frozen at - 20° C.

2.3 Samples Preparation for Protein Quantification

HT29 and 3T3 were subjected to lysis with mammalian cell lysis Kit (SIGMA, product code MCL1).

After the frozen sample cells were thawed at room temperature before lysis. Care was taken to ensure homogeneity of the samples. The samples of HT29 and 3T3 cell line were subjected to lysis in SIGMA Mammalian Cell Lysis Kit.

2.4. Determination of Protein in, 3T3and HT29 (Cell Lines) Samples

15 µl of the sample protein extract of 3T3 and HT29 was placed in an Eppendorf[®] tube; the tests were done in duplicate.

50µl precipitant was added to each tube (including the standard curve tube), vortexed briefly and incubated for 2-3 min at room temperature. 500µl co-precipitant was added to each tube and briefly vortexed. The tubes were centrifuged at 10,000 x g for 5 min, and then removed. The supernatants were decanted and immediately centrifuged and all the remaining water was removed from the pellet using micropipette. The pellet contained the proteins.

Copper solution (100 ml) and 400 µl of distilled water were added to each Eppendorf[®] tube, vortexed briefly to dissolve the precipitated protein. Working color reagent I ml was added to each Eppendorf[®] tube, mixed on a vortex shaker and incubated at room temperature for 15- 20 min.

The absorbance of each sample and standard was read at 480 nm using water for blank as a reference. The absorbance readings were taken within 40 minute after addition of the working color reagent.

Standard curve of BSA was drawn on Microsoft Excel[®]. It depends on the relationship between standard (BSA) concentrations and absorbance reading in a spectrophotometer. Unknown samples of protein concentrations were calculated. The preparation was summarize as shown in Table 1.

Table 1: Method of quantification of protein for 3T3, HT29 by spectrophotometry

Sample	HT29	3T3
Sample Volume / μ l	15	15
Precipitant / μ l	500	500
Vortex and incubate 2-3min at RT		500
Co precipitant	500	
Mix briefly by vortex mixer Centrifuged at 10.000 rpm for 5 min Take pellet after decanting supernatant Add 400 μ l Dist.H ₂ O + 100 μ l Copper solution Vortex Briefly to dissolve the precipitated protein Added 1ml of working color reagent to each tube mix by vortex The samples mixed for few second and incubated at room temperature for 15- 20 min. Absorbance of each sample and standard at 480 nm was read	500	500

2.5. SDS-PAGE

2.5.1. Materials and Apparatus for SDS- PAGE

The materials and chemicals for SDS-PAGE were of analytical grade and includes acrylamide (R & Mchemicals), N'N'-bis-methylene-acrylamide, electrophoresis (Sigma).

SDS (Fisher chemicals), deionized water 18.2 M Ω -oM (Mega Om), Tris base (R & M chemicals), 1.5 M Tris-HCL 8.8 (R & M Chemicals), 0.5 M Tris- HCL 6.8(R & M Chemicals), 6 NHCL (Labsan), glycerol (R& Mchemicals), bromo phenol blue(R & M chemicals), B-Mercaptoethanol (Sigma Aldrich), glycine (Fisher chemicals), ammonium persulfate (R&M chemicals), TEMD (Amersham Biosciences), NaOH (sodium hydroxide) (System®), Laemmli sample buffer (SDS reducing buffer)(Bio Rad), protein extract from 3T3 and HT29, 0.25% Coomassie brilliant blue R- 250 (Biochemical), protein ladder (protein maker) (BIO RAD Precision plus) and protein Kit 2-D Quant Kit (Amersham Biosciences).

The apparatus for SDS-PAGE were of analytical grade and includes electrophoresis (Biorad®), 3 cell assembly and sample loading (Mini-PROTEAN), pH Meter (HI 223 Calibration check Microprocessor), balance (Horizon Land Bhd.), water bath (Memmert), magnetic stirrer, pipette and beakers.

Electrophoresis was carried out in accordance with manufacturer's advice (Bio RAD). For isolation of protein, 12% SDS poly acrylamide gel was prepared according to manufacturer's advice (Mini -PROTEAN®) 3Cell, Bio- Rad. The buffer system was used as described in (Laemmli, 1970), as wet electrophoretic transfer, gave acceptable results in short time (as little as 4 hours) (Towbin *et al.*, 1979). SDS-PAGE was used to separate supernatant substances to produce short peptide chains that tend to elicit antibodies which recognized only the denatured form of the protein (Boersma *et al.*, 1988).

2.5.2. Preparation Protein Samples for SDS-PAGE

After the samples were stored at -20 C° and latter subjected to preparation of protein sample for SDS-PAGE. The Lysis of cells was done by using

mammalian cell lysis (RIPA) buffer Kit (SIGMA, product code MCL1). The sample was vortexed in vortex shaker for 5 minute and then centrifuged to pellet the cellular debris. Protein rich supernatant was removed and decanted the pellet. Quantification of protein concentration was done using Amersham biosciences 2-D Quant Kit. The protein samples from cell lines then take in uniform concentration with Laemmli sample buffer (Bio-Rad®) as described by (Laemmli, 1970).

Samples were boiled to denature protein of antigen before loaded in electrophoresis 12 % SDS-PAGE. The main purpose of denaturing of antigen protein was to produce short peptide with straight chains that tend to elicit antibodies which recognized only the denatured form of the protein (Boersma *et al.*, 1988). Poly acrylamide gel was used for the process of electrophoresis (Towbin *et al.*, 1979); the time duration for electrophoresis was 90 minute at 120 Volts.

2.6. Western Blot

2.6.1. Chemical Materials and Apparatus

The materials and chemical for western blot were of analytical grade and includes 25 mM Tris -HCl (R&M Chemicals), 192 m M glycine (Fisher chemicals), 20% methanol (Sigma), distilled water (Barnstead), Tris base (R&M Chemicals), NaCl (R&M Chemical), Tween 20LABCHEM / AJAX CHEMICALS and skimmed milk.

The reagents for western blot were of analytical grade and includes (IBMR3) supernatant (hybridoma supernatant), secondary antibody (rabbit anti -mouse IgS (H+L) - HRP & goat anti -mouse Igs-horseradish peroxidase (HRP) (ZYMED) Invitrogen immunodetection, Diamino benzyl Tetra hydrochloride (DAB) substrate (Invitrogen) and Ponceau S stain (Sigma).

The apparatus for western blot also were of analytical grade and includes Mini Trans Blot Electrophoretic Transfer Cell (BIO RAD), two filter papers (Invitrogen), two-fiber pads (Invitrogen), pH Meter (Hanna Instrument), magnetic stirrer, PVDF membrane (Invitrogen) and the Belly Dancer shaker (Stovil Greensboro NC USA).

2.6.2. Western Blot

The protein bands on SDS-PAGE for 3T3 and HT29 cell lines, were transfer to Polyvinylidene Difluoride (PVDF) membrane according to Kaspar protocols (Kaspar, 1997). The transfer was performed at 200 mA for 110 minute depending on the size of protein molecules.

2.6.2.1. Immuno Blotting

The membrane was blocked with skimmed milk over shaker then incubated with primary IBMR3 mouse mAb and then incubated with secondary antibody: rabbit anti-mouse immunoglobulin IgS labeled with peroxidases incubated with Di amino benzyl dihydro chloride (DAB) substrate until the brown color appears. On the appearance of the brown color the membrane was washed with water to stop the reaction from further proceeding.

2.6.3. Bio Imaging Machine

The membrane was placed in the Bio Imaging machine under normal light. The analysis of the specific IBMR3 antigen band was done to get the molecular

weight, peak height and raw volume for the molecular band of IBMR3 Ag by densitometry.

2.6.4. Negative Control Mouse IgM

Mouse serum IgM was used as negative control for the IBMR3 antigen, concentration 2.5 mg/ml (lot number 51202537, ZYMED, USA).

3. RESULTS

3.1. Detection of IBMR3 Antigen in 3T 3 and HT29 Cancer Cell Lines

3.1.1. The Results of 3T3 and HT29 Cancer Cell Lines of IBMR3 Antigens

3T3 and HT29 cancer cell lines of IBMR3 antigens proteins were analysis; the concentrations were quantified for samples using the same experimental conditions with same concentrations. The absorbance of samples and optic density were detected using a spectrophotometer. The concentration for the protein of 3T3 (2.569) µg/1µl and HT29 (3.328) µg/1µl as in Table 2.

Table 2: Spectrophotometer reading of standard BSA sample

Number of Sample	BSA volume µl	Concentration 2µg / µl	O.D. Spectrophotometer reading
1	0	0	0.798
2	5	10	0.777
3	10	20	0.684
4	15	30	0.605
5	20	40	0.525
6	25	50	0.464

BSA= Bovine serum albumen, OD = Optical density

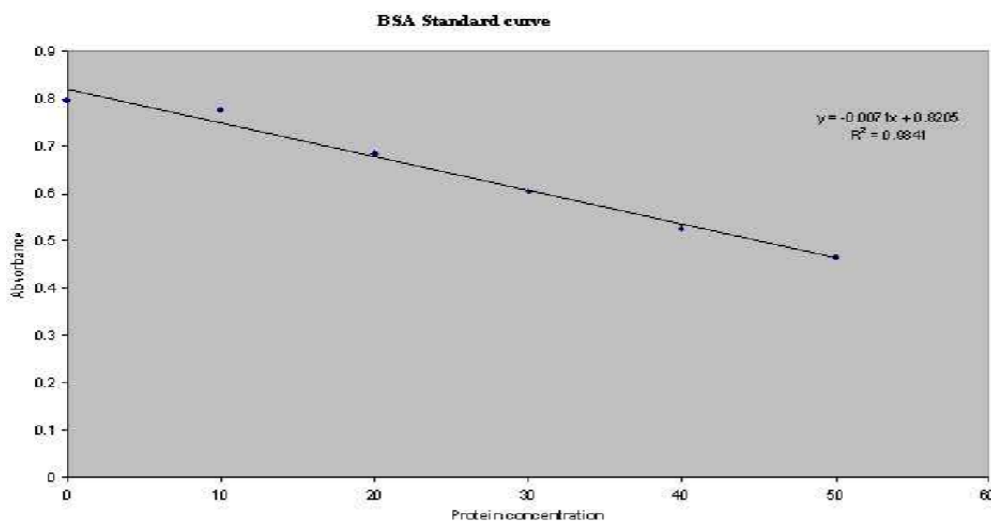


Figure 2: BSA standards curve

Table 3: Unknown cell line samples spectrophotometer absorbance and protein concentration reading

Rank of conc	Samples cell lines	1 st .OD reading A.	2 nd .OD reading A	Mean reading
1	HT29	0.562	0.370	0.466
2	3T3	0.500	0.447	0.4735

OD: optic density

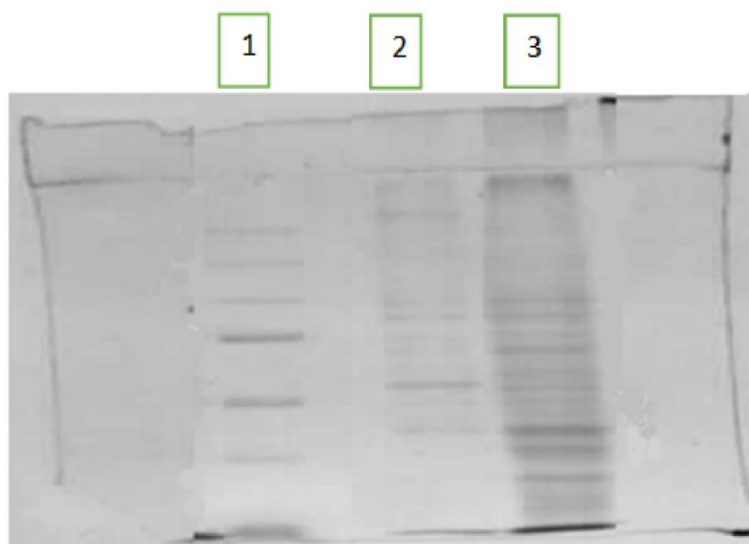


Figure 3: Gel electrophoresis for densitometry analysis (HT29 and 3T3) IBMR3 Ag. 1(PM), 2 (3T3), 3 (HT29)



Figure 4: Cancer cell line IBMR3 Ag: Western blot (1) HT29, (2) 3T3, (3) Protein marker bands on PVDF membrane

Equal concentrations of proteins from each sample were subjected in wells and separated by SDS-PAGE 12% as in Figure 3, the bands were transferred on to PVDF membrane, and probed with mAb IBMR3. The expressions profiles of the antigens recognized by the antibody were different showing the cell line band samples under bio imaging machine as in Figure 4.

3.1.2. Results of 3T3 and HT29 Samples for Negative Control

Negative protein staining for 3T3 and HT29 samples were done by using mouse IgM serotype with secondary antibody rabbit anti-mouse IgG conjugated with (HRP) horse radish peroxidase as in (Figure 5), IgM serotype were not indicated that means no specific antigens for negative control IgM, these findings authenticate the results mention in figure 3 that prove all immunoblotting connections between IBMR3 mAb with specific of antigen epitopes in different antigens in 3T3 and HT29 cancer cell line (Wisdom, GB, 2005).

The expression of IBMR3 antigens in each band of two samples were analyzed by densitometry. Different results were observed for each band that included the molecular weight, peak height and raw volume, as shown in Table 4 and Figure 6.

HT29 IBMR3 Ags showed four different significant bands of MW (90.11, 41.31, 23.87, and 20.86) kDa.

3T3 IBMR3 Ags showed four different significant bands of MW (299.58, 87.90, 41.67, and 23.54) kDa.

The first band for 3T3 cell line regards the significant value of molecular weights between the bands of two samples has the highest molecular weight of (299.58) kDa.



Figure 5: PVDF negative control only (PM) protein marker was indicated

Table 4: Densitometry analysis of IBMR3 Ag in 3T3 and HT29 cell line samples

		1	2	3	4
3T3cell line	MW(KDa)	299.58	87.90	41.67	23.54
	Raw volume	1460168.75	206078.47	161406.89	219583.16
	Peak height	1856.985	551.769,	394.164	216.185
HT29cell line	MW (KD a)	90.11	41.31	23.87	20.86
	Raw volume	110197.11	76106.84	98632.59	221395.34
	Peak height	281.544	101.711	202.668	757.213

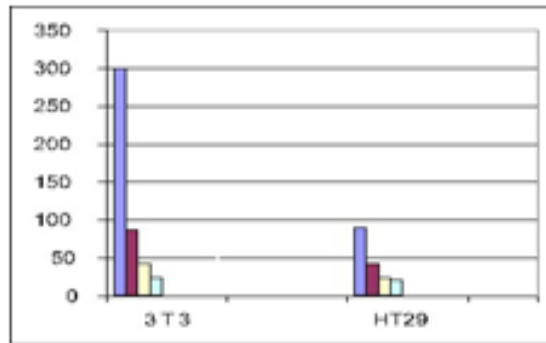
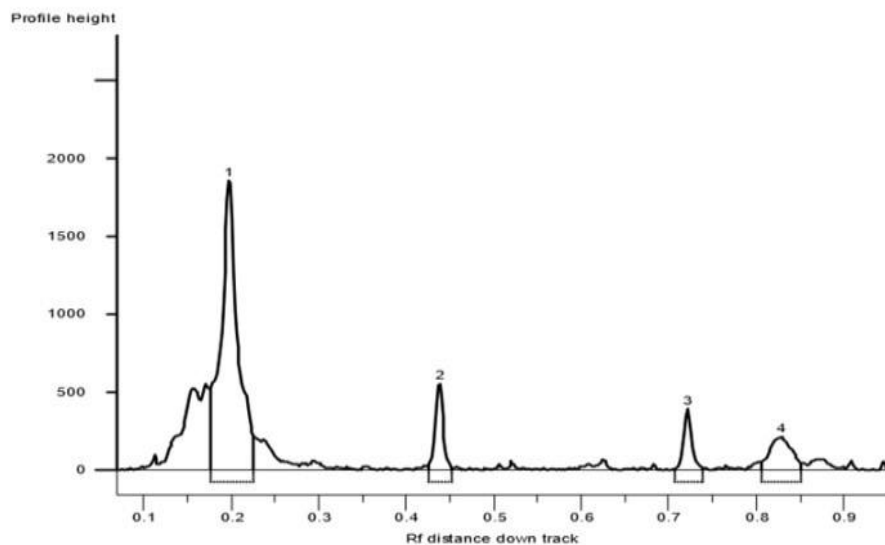


Figure 6: Densitometry analysis of molecular weights IBMR3 Ag with 3T3 and HT29 cell line

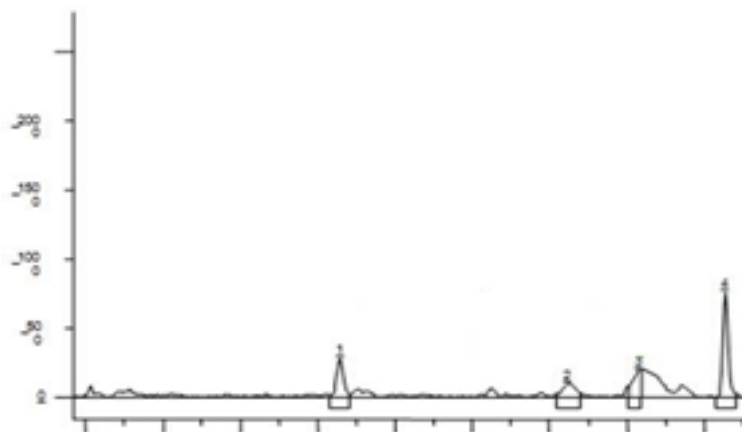
3.1.3. Peak Height Graphs of 3T3 and HT29 IBMR3 Antigens

(Graph d) 3T3, with four characterized peak heights (1856.985, 551.769, 394.164, and 216.185) the

highest peak height was in number 1, (1856.985), further the highest from all the peak height in HT29 sample as shown in Table 4, Graph (d) and Figure 6.



Graph d: 3T3 densitometry



Graph e: HT29 densitometry

(Graph e) HT29, with four characterized peak heights (281.544, 101.711, 202.668, and (757.213). The

highest peak height in number 4 with (757.213). As in table 4, Graph e, and Figure 7.

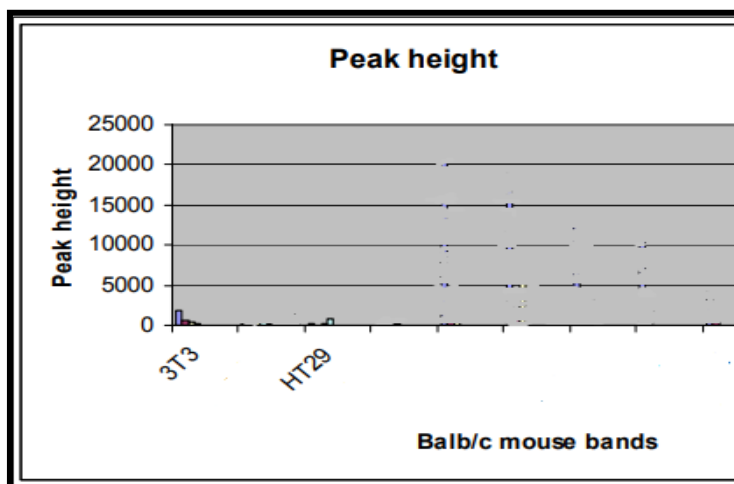


Figure 7: Densitometry of 3T3 and HT29 protein samples for peak height with cell lines in PVDF membrane

3.1.4. 3T3 and HT29 Densitometry of IBMR3 Antigens

3.1.4.1. Raw Volume (Protein Band Concentration)

The results of raw volume for cell lines can be seen in four bands in Table 4, and Figure 8 have shown the value of raw volume (The amount of protein presents in the band).

The greater (contains more protein concentration) was the height concentration in the first band of 3T3 cells (1460168.75) and the lowest concentration was (76106.84) in the second band of HT29.

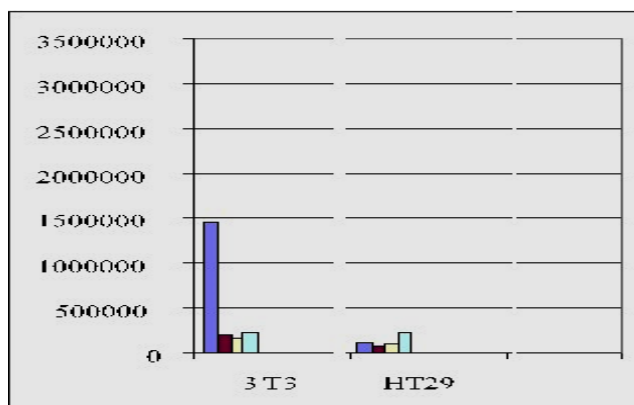


Figure 8: Analysis of cell lines IBMR3 Ag raw volume (protein concentrations) in densitometry

4. DISCUSSIONS

This is the first study to determine and analyze specific IBMR3 antigen level in 3T3 and HT29 cell lines. There is a possibility that mAb IBMR3 may be binding to similar molecules. The variation in the relative molecular weight of the IBMR3 antigens in these two samples indicated that the recognized antigens may be the different domains of the same molecule, or the same liner domain present on different types of molecules (Mat, 1992): (Galizzi *et al.*, 1990).

Nevertheless mAb IBMR3 is proven as a useful reagent for the study of cells. The different values in molecular weights of cell lines may be due to the relation between glycosylation reactions in the antigen and antibody protein cells membrane (Paulick and Bertozzi, 2008) glycosylation has relation of connection between antibody and antigen membrane (Mat, 1992): (Galizzi *et al.*, 1990): (Ferguson and Williams, 1988) : (Kinoshita and Inoue, 2000).

5. CONCLUSIONS

The results from this study suggest that the IBMR3 antigens were expressed in both 3T3 and HT29. However, the expression pattern varied from sample to sample. This might be indicative of different expression profiles of this antigen, as recognized by the antibody that gave different reading in the IBMR3 expression, because results showed different molecular weight in different bands in samples. This might also be indicative of the fact that IBMR3 antibody can express or recognize the same epitopes in different molecules having different relative molecular masses.

6. RECOMMENDATIONS

MAb IBMR3 have potential to be useful for identify and study the roles of these molecules in cancers tissue. This expression profile can be applied to human tissue to detect more specific antigens that might help to use IBMR3 as a marker for cancer diagnosis in the future.

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